

The *ocs* element: a 16 base pair palindrome essential for activity of the octopine synthase enhancer

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A 176 bp DNA sequence lying upstream of the octopine synthase (*ocs*) promoter, previously shown to have enhancer-like properties in transgenic tobacco [Ellis *et al.* (1987) *EMBO J.*, 6, 11–16], functions as an enhancer in protoplasts of *Zea mays* (a monocot plant) and *Nicotiana plumbaginifolia* (a dicot plant). We have characterized this element by transient expression assays using a linked alcohol dehydrogenase (*Adh1*) promoter from *Z. mays* and the chloramphenicol acetyltransferase coding sequences. The *ocs* sequence functions in both orientations but its enhancing activity is dependent upon its distance from the *Adh1* promoter. Transient expression assays using deletion mutants and synthetic oligonucleotides show that a 16 bp palindrome ACGTAAGCGCTTACGT, contained within the 176 bp fragment, is essential and sufficient for enhancing activity in transient expression assays.

Key words: octopine synthase enhancer element/*Adh1* promoter/transcription signals/plant protoplasts

Introduction

Enhancers are DNA sequences that increase the level of transcription from adjacent promoters. They are *cis*-acting DNA elements that are active in either orientation and in many cases can activate transcription when placed several thousand base pairs away from a promoter (Banerji *et al.*, 1981; Fromm and Berg, 1983). Enhancers have been most extensively characterized in the genomes of mammalian viruses and also within animal cellular genomes (reviewed in Gluzman, 1985).

Sequence elements with enhancer-like properties have recently been described in several plant gene promoters. Timko *et al.* (1985) described a large DNA fragment isolated from the promoter of a ribulose-1,5-bisphosphate carboxylase small subunit gene of pea which acted as a light-inducible enhancer. A second light-inducible enhancer element has been described in a 257 bp fragment of a chlorophyll *a/b* binding protein gene isolated from the same species (Simpson *et al.*, 1986). We have reported a 176 bp element isolated from the octopine synthase gene (*ocs*) which has enhancer-like properties (Ellis *et al.*, 1987) in transgenic tobacco plants. These plant gene elements act independently of their orientation and can activate the expression of heterologous promoters.

The *ocs* gene encodes the enzyme involved in the biosynthesis of octopine from arginine and pyruvic acid in crown-gall tumours (reviewed by Tempé and Goldman, 1982). The gene was first isolated from the tumour-inducing plasmid of the plant pathogenic bacterium, *Agrobacterium tumefaciens* (De Greve *et al.*, 1982); it is part of the T-DNA which is transferred from *A. tumefaciens*

to plant cells during tumorous transformation. This gene, although originally isolated from a prokaryote, is only expressed after its introduction into plant cells and thus must contain eukaryotic transcription signals (De Greve *et al.*, 1982).

In transgenic plants the *ocs* gene is expressed constitutively in all tissues and is not under any known environmental or developmental control (Otten *et al.*, 1981). Koncz *et al.* (1983) have shown that sequences between –116 and –292, relative to the start of transcription of the gene, are essential for *ocs* expression in tumours. In a previous report (Ellis *et al.*, 1987) we described some of the properties of this 176 bp region when assayed upstream of the promoter of the maize alcohol dehydrogenase-1 (*Adh1*) gene. In the absence of the *ocs* segment the *Adh1* promoter function in transgenic tobacco plants was barely detectable. Addition of the 176 bp *ocs* segment markedly increased transcription from the normal *Adh1* transcription initiation point; furthermore the *ocs* element acted independently of its orientation with respect to the maize promoter and so exhibited properties similar to enhancer elements of animal genes. In this report we have further defined the enhancer properties of the *ocs* element using a transient expression assay in plant protoplasts.

Results

The ocs element functions in transfected maize protoplasts

Several hybrid promoter constructions were linked to the chloramphenicol acetyltransferase (*cat*) gene (Figure 1) and introduced into maize protoplasts by electroporation for transient expression assays. The level of CAT enzyme expression was assayed as a measure of promoter activity. Construct 1 (pACN-100, *Adh:cat:nos*) contained a 206 bp fragment of the maize *Adh1* promoter (–100 to +106). This construct gave no significant CAT enzyme activity in transient assays in maize protoplasts (Figure 2), since it lacked upstream sequences (–140 to –100) necessary for both the constitutive expression and anaerobic regulation of the *Adh1* gene (Walker *et al.*, 1987). However addition of the 176 bp fragment of the *ocs* gene promoter adjacent to and 5' of the deleted maize promoter (construct 2, pOACN-100, *ocs:Adh:cat:nos*) resulted in substantial CAT enzyme activity; the level of CAT enzyme activity was approximately 200-fold above the level observed in maize protoplasts transfected with pACN-100 (Figure 2). CAT enzyme activity was 5-fold higher than that achieved with the control plasmid p35SCN (Walker *et al.*, 1987) which contains bases –427 to +1 of the strong constitutive 35S promoter of the plant DNA virus CaMV (Figure 2).

In transgenic tobacco the 176 bp *ocs* element functioned independently of its orientation with respect to the *Adh1* promoter (Ellis *et al.*, 1987). When a shorter but functional *ocs* sequence (–116 to –260) was linked in an inverted orientation to pACN-100 to create construct 3 (pINV-Δ5'260, Figure 1) enhancing activity was evident (Figure 2), indicating that enhancing activity in the transient expression assay in maize protoplasts was also independent of orientation.

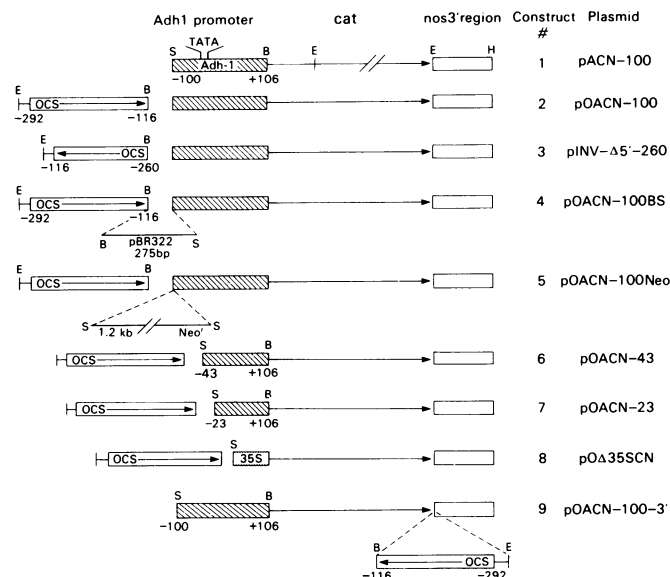


Fig. 1. Chimeric gene constructs used to transflect plant protoplasts were cloned in pUC19 or pUC8. Promoter regions were derived from the maize gene *Adh1* and the 35S gene of CaMV as described by Walker *et al.* (1987). The orientation of the *ocs* DNA is indicated by an arrow which indicates the 5'–3' orientation in the *ocs* gene. The base numbering of fragments refers to the transcription start site (+1) of the respective promoters. Symbols: E = *EcoRI*, B = *BamHI*, S = *SalI*, P = *PstI*, H = *HindIII*, TATA indicates the TATA box region of the *Adh1* gene, *cat* is the coding region of the chloramphenicol acetyltransferase reporter gene, *nos3'* is the region of the nopaline synthase gene containing 3' end processing signals, pBR322 refers to the 275 bp *BamHI* to *SalI* fragment of pBR322, and Neo is the 1.2 kb *SalI* fragment from pLGV1103neo. Unless otherwise indicated, the maize *Adh1* promoter fragment extended from –100 to +106, the 35S promoter extended from –45 to +1, and the upstream region of the *ocs* gene extended from –292 to –116.

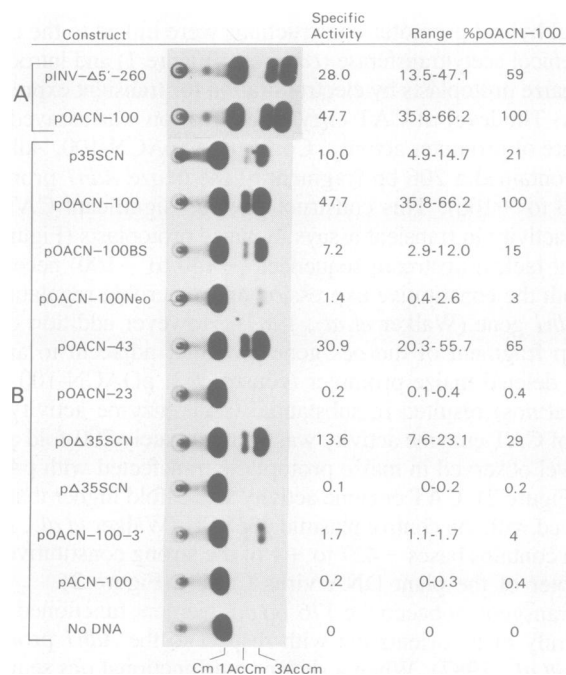


Fig. 2. CAT assays of extracts of maize protoplasts transfected with various chimeric gene constructs. [14 C]Chloramphenicol (Cm) and acetylated reaction products (1AcCm and 3AcCm) were separated by t.l.c. and detected by autoradiography. Specific activity is expressed as c.p.m. of acetylated [14 C]chloramphenicol per μ g of protein less the background observed in protoplasts that were mock-transfected with no DNA. The data presented are the mean of three to five experiments using different protoplast preparations. Parts A and B are from separate experiments.

The effect of the *ocs* element diminishes with increasing distance from the promoter

To determine the effect of altering the distance of the *ocs* element from the transcription start point, insertions of 275 bp and 1.2 kb were placed between the *ocs* and *Adh1* sequences of pOACN-100. Insertion of 275 bp (pOACN-100BS) decreased the activity to 15% that of pOACN-100, and insertion of 1.2 kb (pOACN-100Neo) decreased CAT activity to 3% (Figure 2). In the absence of the *ocs* sequences the spacer DNAs had no enhancing activity of their own (data not shown), indicating that the *ocs* enhancer can act at varying distances from the promoter, although its effect diminished significantly with increasing distance.

To test the effect of increased proximity to the transcription initiation site, the *ocs* element was placed in front of two 5' deletion derivatives of pACN-100. The first deletion [construct 6, pOACN-43 (Figure 1)] placed the *ocs* sequence 10 bp 5' of the first T residue in the TATA box of the maize *Adh1* promoter. The second deletion [construct 7, pOACN-23 (Figure 1)] removed the TATA box region completely. The expression of pOACN-43 was 65% that of pOACN-100, but removal of the TATA box region eliminated all expression. The *ocs* sequence therefore required a functional TATA box for efficient enhancement of gene expression. There was no specific requirement for the *Adh1* TATA box since the TATA region of the 35S promoter of CaMV functioned equally well. In construct 8 (pOΔ35SCN, Figure 1) the 176 bp *ocs* sequence was placed 15 bp upstream of the T residue of the TATA box of a deletion derivative of the 35S promoter. The addition of the *ocs* sequence to the non-functional 35S promoter deletion caused a large increase in promoter activity which was equivalent to the activity of the original 35S promoter construct, p35SCN (Figure 2).

The *ocs* enhancer functions 3' of the coding region

A feature of some viral and animal enhancers is their ability to activate transcription when they are positioned 3' of the gene (Banerji *et al.*, 1981; Fromm and Berg, 1983). We tested a construct where the 176 bp *ocs* enhancer element was positioned between the *cat* gene coding region and the *nos* 3' end processing signals to test whether the *ocs* enhancer would work 3' of this reporter gene (construct 9, pOACN-100-3'; Figure 1). The plasmid DNA was introduced into maize protoplasts as either circular molecules (Figure 2) or linearized at the unique *HindIII* site at the 3' end of the chimeric gene (data not shown). Under both conditions CAT activity was only 4% that of pOACN-100; nevertheless the presence of the *ocs* enhancer increased CAT enzyme activity approximately 9-fold above the background, indicating that it can function 3' of the *cat* gene coding region. The lower level of CAT expression in pOACN-100-3' was not due to insertion of sequences between the CAT coding region and the *nos* gene 3' end processing region. The presence of an extra copy of the *ocs* DNA inserted at the same position in pOACN-100 did not decrease CAT activity (results not shown). The level of CAT activity observed probably reflects the distance of the enhancer from the promoter. The low level of enhancement is close to that seen for the construct pOACN-100Neo in which the *ocs* enhancer lies 1.3 kb 5' of the transcription start site; in construct 9 (pOACN-100-3') the *ocs* enhancer was approximately the same distance (1 kb) 3' of the transcription start site.

Deletion analysis of the *ocs* enhancer fragment

Progressive deletions were made to delimit the sequences within the *ocs* fragment responsible for the enhancing activity. Deletions from either the 5' or 3' end of the 176 bp *ocs* promoter fragment were linked to pACN-100 and tested for CAT expression

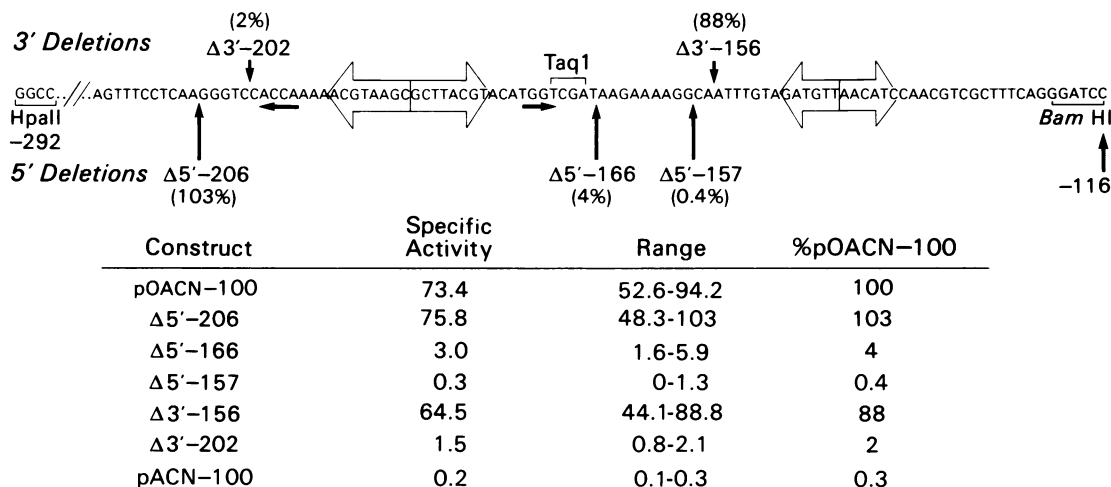


Fig. 3. Sequence of the upstream region of the *ocs* gene promoter from a 5' site (–217) to the 3' *Bam*HI site (–116) from DeGreve *et al.* (1982). The end points of deletions removing DNA from the 3' end are shown by vertical arrows above the sequence, and the end points of the 5' deletions are shown below the sequence. The activities of the deletion constructs in comparison with pOACN-100 are given in brackets, and the data are shown in the table. The specific activity values were determined as described in Figure 2 and are derived from three to four separate experiments. The positions of the 12 bp and 16 bp palindromes are indicated by open arrows. The short inverted repeat at the ends of the 16 bp palindrome is indicated by horizontal arrows.

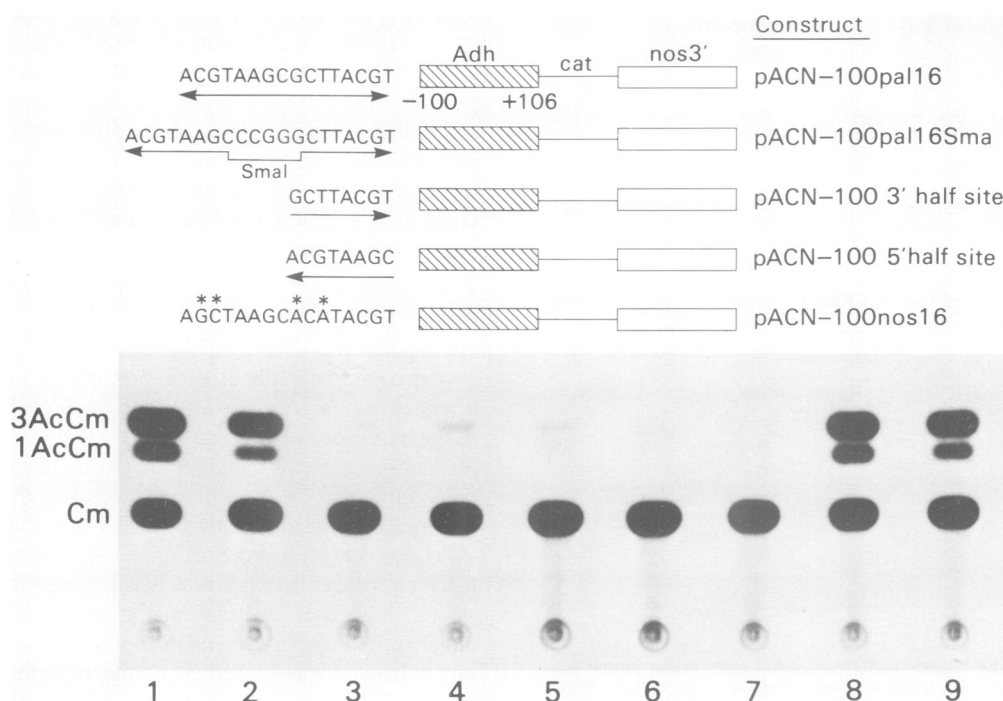


Fig. 4. Structures of chimeric gene constructs containing a synthetic 16 bp palindrome and its mutant derivatives and the CAT activities in extracts of maize protoplasts transfected with these plasmids. The numbers represent the following plasmids. 1 = pOACN-100; 2, 8 and 9 are replicate experiments using pACN-100pal16; 3 = pACN-100pal16-Sma; 4 = pACN-100 5' half-site; 5 = pACN-100 3' half-site; 6 = pACN-100; 7 = no DNA. The asterisks show where the sequence of the *nos* 16-mer differs from the 16 bp palindrome. The synthetic oligonucleotide duplexes were cloned into the *Eco*RI and *Bam*HI sites in the polylinker of pUC19.

following electroporation into maize protoplasts. Deletion end points of critical constructs and enhancer activity in comparison with pOACN-100 are indicated in Figure 3. Deletion of 86 bp from the 5' end to position –206 (mutant $\Delta 5'-206$) had little effect on enhancer activity. Removal of an additional 40 bp (mutant $\Delta 5'-166$) decreased enhancer activity to 4%. The sequences responsible for the enhancer effect were further defined by deletions from the 3' end. Mutant $\Delta 3'-157$ had 88% of full activity, but an additional deletion of 46 bp (mutant $\Delta 3'-202$) resulted in only 2% activity. Taken together the results of 3' and 5' deletions

defined a 50 bp region (–206 to –156) that is essential for the majority of the *ocs* enhancer effect in maize protoplasts. A structural feature of the DNA sequence within this region is a 16 bp palindrome ACGTAAGCGCTTACGT from –193 to –178. This sequence is flanked by a 4 bp inverted repeat (Figure 3) which occurs 3 bp beyond the ends of the palindrome. A second palindrome of 12 bp, GATGTTAACATC, occurs between –148 and –137. Deletion mutants ($\Delta 3'-156$ and results not shown) demonstrated that the 12 bp palindrome contributed little if any to the *ocs* enhancer activity in maize protoplasts.

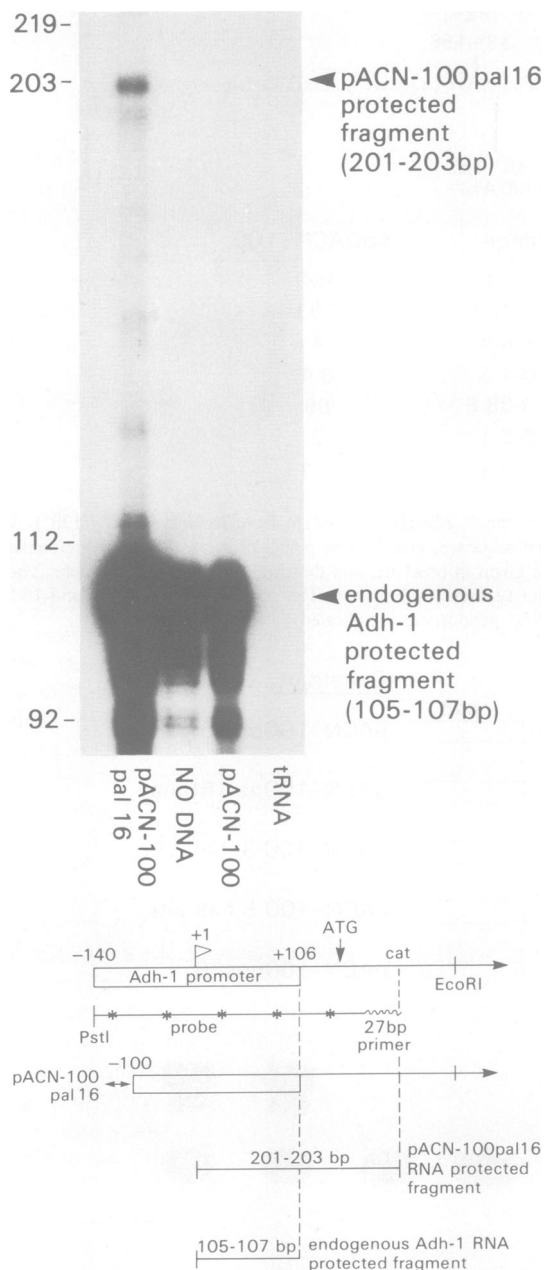


Fig. 5. S1 analysis of the 5' ends of RNA isolated from maize protoplasts transfected with chimeric gene construct pACN-100pal16 or pOACN-100 and of the 5' end of RNA transcribed from the endogenous *Adh1* gene present in the genome of BMS maize. Controls were S1 analysis of RNA extracted from protoplasts that were mock-electroporated and S1 analysis using tRNA. 50 μ g of total RNA was used in the pACN-100pal16 lane and 25 μ g in other lanes. The probe was uniformly 32 P-labelled single-stranded DNA synthesized from a single-stranded M13 clone of the chimeric *Adh/cat* gene pACN-140 (Walker *et al.*, 1987), extended from a 27 base primer homologous to a region of the *cat* gene (Howard *et al.*, 1987). Symbols: P = transcription start; ATG = translation initiation codon and \longleftrightarrow represents the 16 bp palindrome. The structure of pOACN-100pal16 is shown in Figure 4.

The 16 bp palindrome is sufficient for enhancer effect

The 16 bp palindrome was synthesized and inserted into the test plasmid pACN-100 (Figure 4) to give pACN-100pal16. This synthetic sequence was sufficient to enhance the expression of the inactive *Adh1* promoter in transient gene expression analysis, and its activity ranged from 50 to 70% of that of the 176 bp *ocs* sequence. Although the major enhancing activity in transient

assays was due to the 16 bp palindrome, either distance of the element from the promoter or the presence of surrounding sequences in the 176 bp fragment may have some effect on the final level of enhancement.

Several variants of the 16 bp sequence were synthesized as part of an initial mutation study of the palindrome. In the first the 4 bp sequence CCGG was inserted to create a *SmaI* restriction site in the centre of the palindrome. This 4 bp insertion which created a 20 bp palindrome eliminated all enhancing activity (Figure 4). From this mutant two deletion mutants were made. The first deletion removed the 8 bp of the 5' half of the palindrome and the second, the 8 bp of the 3' half. Neither 8 bp 'half-site' had an enhancing activity (Figure 4). A fourth variant of the *ocs* element provides information on the level of sequence alteration that the palindrome can sustain before enhancing activity is lost. Four bases within the palindrome were varied to create the synthetic sequence AGCTAAGC^{*}ACATACGT (changes indicated by *). This particular sequence variant of the *ocs* element was studied because it occurs 5' of the promoter of the nopaline synthase gene (*nos*) in a 29 bp region (-130 to -101) that is essential for activity of the promoter (An *et al.*, 1986). Like the *ocs* gene, *nos* occurs in the T-DNA of *A. tumefaciens* and is transferred to plant cells where it is expressed constitutively (De Block *et al.*, 1984). A synthetic oligonucleotide containing the *nos* gene 16-mer was inserted into pACN-100 to give the construct pACN-100nos16 (Figure 4), such that its orientation was identical to that in the *nos* gene. Maize protoplasts electroporated with pACN-100nos16 expressed no CAT enzyme activity above the level of pACN-100 (result not shown). Therefore the 4 base changes which destroyed the symmetry of the *ocs* element also eliminated enhancing activity.

The *ocs* enhancer functions in *Nicotiana plumbaginifolia* protoplasts

All experiments described above used maize protoplasts. Parallel experiments were done using protoplasts of the dicot plant *N. plumbaginifolia*. The results of these experiments were similar to the maize protoplast experiments. Deletion analysis and oligonucleotide synthesis identified the 16 bp palindrome as the essential component of the *ocs* enhancer (results not shown). One difference between the two species was that expression of the *Adh1* promoter was more sensitive to position of the *ocs* enhancer. CAT enzyme expression was barely detectable when the 275 bp spacer was inserted between the *Adh1* promoter and the *ocs* enhancer. Furthermore enhancement was not detected when the *ocs* enhancer was placed 3' of the reporter gene.

Analysis of transcription in electroporated protoplasts

Before ascribing the role of an enhancer to a sequence it is important to determine that no promoter functions are contributed by the sequence. The fact that the TATA box of the maize *Adh1* promoter was required for expression indicated that the *ocs* enhancer did not function as a promoter. In this section we describe an experiment which shows that the 16 bp palindrome does not alter the transcription start of the *Adh1* promoter segment in maize protoplasts.

Previously S1 analysis of transcripts from the *ocs-Adh1* gene construct introduced into transgenic tobacco demonstrated that initiation occurred at the identical position in the *Adh1* sequence as that used in maize cells (Ellis *et al.*, 1987). Similarly in the transient assays of the *ocs-Adh1* gene constructs in maize protoplasts, we found initiation at the normal maize site. For these studies the construct pACN-100pal16 as introduced into maize protoplasts and RNA isolated. To obtain sufficient RNA for S1

analysis, 3 mg of plasmid DNA was electroporated into 4.5×10^7 protoplasts. These conditions represent a 30-fold increase in the size of the experiment compared with the standard conditions used for a CAT assay. A uniformly labelled single-stranded DNA probe of approximately 340 bases was hybridized to RNA isolated from maize protoplasts that had been electroporated with pOACN-100pal16 (lane 1, Figure 4), or no DNA (lane 2, Figure 4) or pACN-100 (lane 3, Figure 4). The vast majority of the protected transcripts were approximately 106 bases in length and originated from transcription of the endogenous *Adh1* gene in the maize protoplasts. In cells electroporated with pACN-100pal16 a second weaker band approximately 201 bases in length was detected. This band is due to transcription from the fused *Adh1 cat* gene in the pACN-100pal16 plasmid DNA. The length predicted is 201 bases if transcription of the hybrid gene is initiated at the normal maize *Adh1* transcription initiation site (Ellis *et al.*, 1987; Howard *et al.*, 1987). No protected fragments longer than 201 bases were detected in cells electroporated with pACN-100pal16. Therefore the 16 bp palindrome does not alter the normal mRNA transcription site of the transfected chimeric gene.

Discussion

In this paper we have identified a 16 bp palindrome sequence ACGTAAGCGCTTACGT that is an essential component of the *ocs* enhancer. This element occurs between bases -193 and -178 of the octopine synthase gene enhancer. The 16 bp palindrome is part of a larger 176 bp fragment of the *ocs* gene that has been used previously to enhance the expression of the maize *Adh1* promoter in transgenic tobacco (Ellis *et al.*, 1987). We have used transient gene expression analysis in plant protoplasts to characterize the 176 bp *ocs* enhancer. Placement of this element at positions both 5' and 3' of a chimeric maize *Adh1/cat* gene demonstrated enhancer activity, but there was a strong dependence on the distance from the promoter. In pOACN-100, where the 16 bp palindrome occurs at -163 bp with respect to the transcription start point, the *Adh1* promoter activity was increased 200-fold. However when the spacing was increased placing the palindrome at -438 (pOACN-100BS), or at -1463 (pOACN-100Neo), promoter activity in maize protoplasts fell to 15% and 3% respectively of the activity of pOACN-100. The lower level of enhancing activity observed when the *ocs* enhancer was placed 1 kb 3' of the transcription initiation point probably resulted from this same distance effect. In protoplasts of *N. plumbaginifolia* the effect of the distance between promoter and enhancer was more pronounced. This may be due to the fact that the *Adh1* promoter is in a heterologous host species that is distantly related to maize.

Decrease in enhancer activity with distance from the promoter has been observed with animal gene enhancers when associated with certain promoters. For example the long range activity of the *Xenopus hsp70* gene enhancer requires the CCAAT box of the *hsp70* gene (Beinz and Pelham, 1986). In this case the enhancer effect remains constant with increasing distance in the presence of the CCAAT box but diminishes with distance when the CCAAT sequence is disrupted. Similarly the SV40 enhancer requires specific promoter sequences for long range activation of the β -globin gene (Grosveld *et al.*, 1982). Physical interaction between factors binding to the enhancer and CCAAT box (or other promoter elements) has been proposed to account for this distance effect (Beinz and Pelham, 1986).

In transgenic tobacco the 176 bp enhancer fragment functioned in either orientation (Ellis *et al.*, 1987). Similarly a smaller 144 bp

enhancer fragment functioned in plant protoplasts independent of its orientation. The 2-fold symmetry of the 16 bp *ocs* element would account for this property.

In transient gene expression assays in protoplasts, the 16 bp *ocs* element is necessary and sufficient for enhancer activity. Earlier work (Koncz *et al.*, 1983) demonstrated that a 5' deletion mutant to a *TaqI* site at -168 (Figure 3) destroyed the activity of the *ocs* promoter when assayed in stably transformed tobacco tumours. This deletion removed the 16 bp palindrome and indicates that the palindrome may be essential for expression of the *ocs* promoter in stably transformed cells. We are currently testing whether the 16 bp palindrome is sufficient to enhance the *Adh1* promoter in stably transformed plant cells.

Both the integrity of the 16 bp sequence and its dyad symmetry were important for its enhancer activity. Insertion of 4 bp between the halves of the palindrome destroyed its enhancer activity and neither 8 bp half of the palindrome was sufficient for activity. An extensive mutational analysis should reveal the essential features of the 16 bp sequence.

We have searched the sequence of the T-DNA of an octopine Ti plasmid (Barker *et al.*, 1983) and the sequence of the nopaline synthase gene (Depicker *et al.*, 1982; Bevan *et al.*, 1983) for other regions homologous to the *ocs* element. Only one was found in the nopaline synthase gene which, like *ocs*, is constitutively expressed in plant cells. This sequence (Figure 4) which shares 12 out of 16 bases with the *ocs* element did not act as an enhancer in our assay. It is nevertheless possible that this element is part of an important promoter element in the *nos* gene, because it lies in a 29 bp region (-130 to -101) of the *nos* promoter which is essential for promoter activity (An *et al.*, 1986).

Regions of DNA sequence with dyad symmetry that control gene expression have been documented for both prokaryotic and eukaryotic genes. The binding sites of the repressor and *cro* proteins of phage lambda are examples. In each case the sites are 17 bp imperfect palindrome sequences, symmetrical about a central nucleotide; each 'half-site' of the dyad is a binding site for a monomer of the respective dimeric proteins (reviewed by Ptashne, 1986). Fewer dyad sequences controlling gene expression have been described for eukaryotic genes. Examples include the symmetrical 14 bp consensus sequence of *Drosophila* heat shock genes that are recognized by a specific heat shock transcription protein (Parker and Topol, 1984) and also the 9 bp symmetric core consensus sequence of the GCN4 protein binding sites in yeast amino acid biosynthetic genes such as the *his3* gene (Hill *et al.*, 1986). We are presently looking for a DNA binding protein from plant cells that interacts with the *ocs* 16 bp palindrome. Since the *ocs* gene is not present in normal plant cells but is only introduced by transformation by a pathogenic bacterium, such a binding protein might be expected to function in normal non-transformed plant cells. Furthermore because the *ocs* enhancer is functional in both dicot and monocot cells, this protein presumably has a universal function and may occur in all higher plants. If this were the case it would be of interest to know which plant gene or genes share control regions with the octopine synthase gene.

Materials and methods

Plasmids and hybrid gene constructs

The 5' deletion derivatives of the *Adh1* gene promoter (the *Adh1*-1S allele, Dennis *et al.*, 1984) were derived from pAdhCAT (Howard *et al.*, 1987) by *Bal31* deletion as described by Walker *et al.* (1987). The *EcoRI*-*Bam*HI fragment containing the *Hpa*II (-292) to *Bam*HI (-116) portion of *ocs* upstream promoter region (De Greve *et al.*, 1982) was cloned in pUC8 as described by Ellis *et al.* (1987) and deleted in both directions by *Bal31* as described by Walker *et al.* (1987).

An *XhoI* linker (CCTCGAGG, Biolabs) was added to the 5' deletion constructs and a *Sall* linker (GGTCCGAC, Biolabs) was added to 3' deletions. The *Adh1* promoter deletion plasmid pACN-100 was made by Walker *et al.* (1987) and contains bases -100 to +106 of the *Adh1* promoter, the coding region of the bacterial gene *cat* and the 3' processing region of the *nos* gene. The deleted 35S promoter (-45 to +1) plasmid pΔ35SCN was described by Walker *et al.* (1987) and also contained the *cat* gene and *nos* 3' end. To make the spacing mutants the 275 bp *Bam*HI-*Sall* fragment of pBR322 (Bolivar *et al.*, 1977) and the 1.2 kb *Sall* fragment containing a bacterial kanamycin resistance gene isolated from pLGV1103Neo (Hain *et al.*, 1985) were inserted into pOACN-100.

Oligonucleotide synthesis and cloning

Oligonucleotides were synthesized using an Applied Biosystems 380A DNA synthesizer. The 16 bp palindrome was synthesized as two 22 base oligonucleotides, GATCCACGTAAGCGCTTACGTG and AATTCACGTAAGCGCTTACGTG, which were annealed in 10 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 50 mM NaCl, by heating to 100°C in a water bath which was allowed to cool slightly to 25°C. When the two oligomers annealed *Bam*HI and *Eco*RI overhanging ends were created, and the DNA duplex was cloned into pUC19 which had been digested with *Bam*HI and *Eco*RI. The resulting plasmid was pUC19pal16. The plasmid pUC19pal16-Sma was made similarly using oligomers containing the extra 4 base CCGG at the centre of the palindrome. The *Sall*-*Hind*III fragment of pACN-100 (Figure 1) was cloned into these two vectors to give pACN-100pal16 and pACN-100pal16-Sma (Figure 3). The 3' 8 bp 'half-site' deletion of the palindrome was made by cutting pACN-100pal16-Sma with *Sma*I and *Hinc*II (the *Hinc*II site is part of the *Sall* site positioned at -100 of the *Adh1* promoter in pACN-100) and then religating. To make the 5' 8 bp 'half-site' deletion, pUC19pal16-Sma was digested with *Eco*RI (the *Eco*RI site is the 5' boundary of the palindrome) and *Sma*I, then filled in with Klenow fragment of polymerase I and religated. The *Sall* to *Hind*III fragment of pACN-100 was then inserted. pACN-100nos16 was constructed like pACN-100pal16 starting with 22-mers, GATCCAGC-TAAGCACATACGTG and AATTCACGTATGTGCTTAGCTG. The duplex formed from these two annealed oligomers also had an *Eco*RI and a *Bam*HI overhanging end for cloning into pUC19. When the *Sall* to *Hind*III fragment of pACN-100 was inserted to create pACN-100nos16, the orientation of the 16 bp *nos* gene sequence with respect to the *Adh1* promoter was identical to its orientation in the *nos* promoter.

Transient expression analysis

Maize protoplasts were isolated from a suspension culture of the black Mexican sweet (BMS) variety (Chourey and Zurawski, 1981) and *N. plumbaginifolia* protoplasts were isolated from the suspension culture line NpT5 cultured as described by Llewellyn *et al.* (1987). The protoplasts were electroporated with plasmid DNA and assayed for CAT expression as previously described (Fromm *et al.*, 1985; Howard *et al.*, 1987; Llewellyn *et al.*, 1987). Each construct was assayed three or five times using different protoplast preparations, and the assays were quantitated by cutting the ¹⁴C-labelled acetylated forms of chloramphenicol, which are products of the reaction, from t.l.c. plates and counting in a scintillation counter. Specific activities of the enzyme extracts were expressed as c.p.m. of acetylated chloramphenicol per µg of protein.

RNA preparation and S1 nuclease assays

The S1 analysis was carried out as described by Howard *et al.* (1987) using RNA isolated by the method of Freeman *et al.* (1983).

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Note added in proof

During proof reading, we noticed that the 16 base-pair sequence in pACN-100nos16 was inverted with respect to its orientation in the nopaline synthase gene. A new construct in which this synthetic oligomer was placed in the correct orientation was made and tested in maize and *N. plumbaginifolia* protoplasts. This corrected construct gave no significant CAT activity in electroporated protoplasts.